Interspecific Hybridisation and Polyploidy for Creating Novel Genetic Combinations©

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INTRODUCTION

Interspecific hybridisation provides a valuable tool that creates exciting new opportunities for breeding of new plants. In crosses between closely related species there usually will be little or no difficulty in producing hybrids. In this paper we focus on interspecific hybridisation in parental combinations where outcomes may not be as expected for conventional crosses. Interspecific hybridisation differs from conventional crosses in that we are developing novel plant genotypes by overcoming the barriers that separate species. We also discuss the use of chromosome doubling to produce polyploids as a method to restore fertility in sterile hybrids, and a number of techniques used to verify the outcomes of hybridization and chromosome doubling will be described. Not all attempts to hybridise between species will be successful, but if barriers to success can be identified, in many instances there are solutions that will enable the production of a fertile hybrid or the transfer of a desired trait into a backcross hybrid. This paper provides a brief introduction to this topic; a more comprehensive review was recently published by Morgan et al. (2011).

Interspecific hybridisation and polyploidy are recognised as important forces in the evolution of flowering plants. There are many examples of hybridisation being used to improve crop performance. Hajjar and Hodgkin (2007) reviewed the breeding of 19 crop species and identified 13 with cultivars based on interspecific hybrids (60 wild species contributed over 100 useful traits in these crops). Distant hybridisation has even contributed new species to cultivation; triticale is a hybrid of wheat and rye. It is estimated that 15 million tonnes of triticale were harvested in 2009 <http://en.wikipedia.org/wiki/Triticale>. The role of hybridisation with wild relatives in the development of cultivars is probably under-estimated, as such breeding efforts are often poorly documented and parent species poorly described.

The value of hybridisation with wild relatives to crop improvement is clearly considerable, though difficult to accurately quantify. Pimentel et al. (1997) estimated a contribution of $115 billion per year to crop yields worldwide. There has thus been considerable research to better understand hybridisation and hybrids. Exciting new advances in identifying sought-after traits in related species and techniques for generating and characterising hybrids will facilitate future use of hybridisation as a breeding technique.

Interspecific hybridisation can be a natural process where species' distributions overlap, sometimes leading to new hybrid species. *Senecio* species (ragwort or

groundsel) provide a well documented example. Following its introduction to the United Kingdom *S. squalidus* hybridised with *S. vulgaris* resulting in two new species, *S. cambrensis* and *S. eboracensis* (Abbott et al., 2009). Hybridisation, both natural and directed by breeders, can have variable results. Outcomes from closely related species are often fertile hybrids combining traits from both parent species. Infertility, albinism, or "hybrid weakness" are frequent with wider crosses that additionally can be much more difficult to produce. The first successful attempt to create a synthetic hybrid species was ×*Raphanobrassica* by Karpechenko in the 1920s when he crossed radish with cabbage. Unfortunately for Karpechenko, his allopolyploid had the roots of a cabbage and the leaves of a radish.

For natural hybrids to give rise to new species with novel traits, the hybrid must be able to contribute to a next generation either through self pollination, crossing with similar hybrids, or back crossing to either parent. Plant breeders are often not seeking the hybrid, but rather are seeking to transfer (introgress) a trait(s) from one species to another. This requires that the hybrids are at least partly fertile. In sterile hybrids fertility can be restored through chromosome doubling of diploid plants to produce polyploid plants, often tetraploid (with four sets of chromosomes). Chromosome doubling can occur through natural processes or through treating plant tissues with chemicals such as colchicine that prevent chromosome separation at cell division. The "Triangle of U" is a theory on the evolution of a number of *Brassica* species (U, 1935). Essentially, three ancestral species combined resulting in three of the contemporary species, *B. carinata*, *B. juncea*, and *B. napus* (Fig. 1). From 46% to 68% of annual crop species are estimated to be polyploid; this figure is higher for perennial crop species, at 60%–76% (Hilu, 1993). The effects of polyploidy

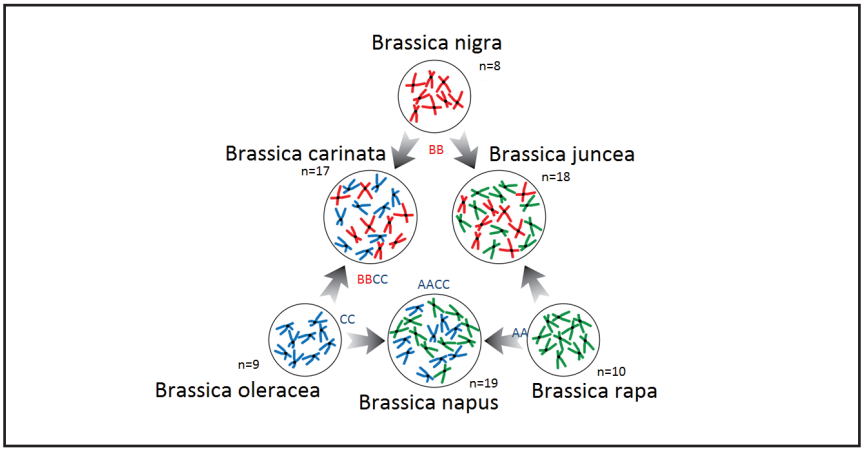

Figure 1. Evolution of cultivated brassicas through introgression of genomes by hybridisation coupled with polyploidy. Three "new" species were derived from three ancestral species though the processes of hybridisation and polyploidy. AA, BB, and CC refer to the genomes of the ancestral species *B. rapa*, *B. nigra*, and *B. oleracea* respectively. The combination AABB of *B. juncea* thus refers to the allotetraploid derived from hybridisation between *B. rapa* and *B. nigra*. More details are at <http://en.wikipedia.org/wiki/Triangle_of_U>.

on plant form, e.g., larger flowers, are such that tetraploids are frequently used as ornamental crop cultivars (it is estimated that 50%–70% of ornamental crops are polyploids [van Harten, 1998]). Chromosome doubling is therefore frequently used as a technique to create new options for ornamental cultivars.

SPECIES

"Species" is a concept which appears very familiar to most of us. It is the basic taxonomic rank in biological classification. Species are usually named using their binomial classification, e.g., *Gentiana triflora,* where *Gentiana* is the genus and *triflora* is the species. The definition of species most of us are probably familiar with is something like "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr, 1942). For the purposes of this discussion we will use this definition and, therefore, developing novel genotypes by overcoming the barriers that define species is the focus of this paper. Additionally, while debate on the nature of species may seem academic it does have practical implications; for instance the New Zealand Plant Biosecurity Index is species based. A similar situation likely occurs for other countries where restrictions on plant imports are made on a species basis, even though there are many genera in which the "species" will hybridise freely and it may thus be difficult to accurately describe species. For example a recent study of cultivated sunflower showed that *Helianthus annuus* is hybridising freely with its wild relative *H. argophyllus* (Heesacker et al., 2009). This situation is further complicated by the "quality" of the definition of the species, with some species being poorly defined.

Species are isolated by barriers to gene flow that occur at different stages of the hybridisation process. These may be referred to as pre-or post-zygotic barriers depending on whether they occur before zygote (fertilised egg before first cell division) formation or following zygote formation. Pre-zygotic barriers act to prevent fusion of sperm and egg cells (zygote formation). These barriers may include one or more of spatial separation of the species, differences in flowering time, pollen transfer mechanisms, and interactions between pollen and both pistil and ovule. All these factors operate to prevent formation of the embryo. There are a range of techniques that have been used to overcome these barriers.

In the event that an embryo is produced, a range of post-zygotic barriers can operate to prevent formation of the hybrid plant, or if a hybrid plant is produced, prevent the new plant contributing to a next generation. The failure of hybrid seed development is usually attributed to failure of endosperm development; this is typically overcome using embryo rescue (embryo or ovule culture). Post-zygotic barriers may include infertility, "hybrid weakness," and albinism, all of which limit the ability of the hybrid to contribute to a next generation.

PROTOCOLS FOR HYBRIDISATION

A wide range of techniques has been successfully applied to overcome different barriers to the production of interspecific hybrids in diverse taxa. There are many publications on hybridisation in *Lilium* (e.g., van Tuyl et al., 1991; van Tuyl and De Jeu, 1997; van Tuyl, 1997; van Tuyl et al., 1997; van Tuyl and Lim, 2003). A similar situation exists for many crops in which hybridisation forms an integral part of breeding programmes. Difficulties in successful distant hybridisation can be divided into pre- and post-zygotic barriers.

Pre-zygotic Barriers. Pre-zygotic barriers that prevent the fusion of gametes and thus formation of the embryo can be manipulated in a range of ways. For example, collection and storage of pollen can provide a cost-effective way to overcome differences in flowering time. For many species storing pollen is reasonably simple using silica gel to dry anthers collected just prior to dehiscence, and freezing. Sukhvibul and Considine (1993) provide details of a protocol for *Anigozanthos*.

There is a complex interaction between pollen and pistil operating to protect the ovule from "inappropriate" pollen, such as that from other species. Many techniques have been used to overcome pollen-pistil barriers, but it is important to recognise that the interaction between pollen and pistil is more than just recognising the "right" pollen and providing a channel for the pollen tube to grow to the ovule. Techniques such as mixed or mentor pollination, use of high temperature or chemical treatments to block enzyme interactions, or cut style-pollination (removing the stigma and all or part of the style then pollinating the cut end) have all been successfully applied. Pollination of isolated ovules, placental pollination, or style grafts have also been used (discussed by van Tuyl and de Jeu, 1997). Vervaeke et al. (2002) experimenting with *Aechmea* found cut style and placental pollination gave lower rates of ovule penetration by pollen tubes than did stigmatic pollination. Improved ovule penetration following cut style pollination was observed when a longer portion of the style was left attached to the ovary. From their experiments Vervaeke et al. (2002) concluded that pollen tube growth through the style was needed to guide pollen tubes to the ovules. The role of the pistil in pollination is thus much greater than simply rejecting the "wrong" pollen, as there is also a role in supporting the growth of the "right" pollen.

Post-zygotic Barriers. Developing seed with hybrid embryos may fail to mature, and fruits may abscise at an early developmental stage. This is usually attributed to failure of endosperm development. Pollination in flowering plants leads to double fertilisation; one sperm cell fuses with the egg cell to produce a diploid embryo and the other sperm cell fuses with the two haploid polar nuclei to produce the triploid endosperm. The endosperm nourishes the embryo in later stages of seed development and/or during seed germination. The endosperm may be entirely absorbed by the embryo before the seed is fully developed, e.g., legumes, or part of the endosperm may remain to be absorbed at germination, e.g., cereals. The embryo begins to utilise endosperm reserves at about 28 days after pollination in *Sandersonia aurantiaca* (seed takes about 60 days to mature) (Zou et al., 2001). In vitro culture (tissue culture) of embryos (embryo rescue) can be used to grow immature embryos to plants. Plants grown aseptically under in vitro culture (tissue culture) conditions are provided all of the nutritional and energy requirements needed for growth and development in their growing media.

Producing hybrid plants by transferring abortive embryos to in vitro culture, commonly termed embryo rescue, has been successfully achieved across a diverse range of genera, for example *Brassica* (Diederichsen and Sacristan, 1988), *Sandersonia* (Burge et al., 2008), *Gentiana* (Morgan, 2004), or *Solanum* (Jansky, 2006). With the advancements of in vitro techniques, embryo culture following interspecific hybridisation has become an important application of this technology. Embryos, isolated or within ovules, are introduced to in vitro culture within days or weeks of pollination. As a rule of thumb, it is best to leave the embryos to develop on the plant for as long as possible. In general, the earlier the rescue is attempted the more

difficult it is to regenerate plants from the isolated embryos, and the more complex the needs of the immature embryo. Embryos may develop "normally" and grow into normal, healthy plants. In other hybrids, e.g., some crosses between various *Limonium* species, embryos will begin to grow then die (Morgan et al., 1998). This barrier can be overcome by using plant growth regulators to induce formation of a hybrid callus, from which plants can later be regenerated. The key to success is to maintain growth of the hybrid plant tissue, and inducing callus formation provides a mechanism for doing this. Embryo culture was used to rescue abortive embryos from inter-generic crosses between *Sandersonia aurantiaca* and both *Littonia modesta* and *Gloriosa superba* (Burge et al., 2008). Thus embryo rescue may be the only way for recovery of plants from some wide crosses.

There can be a considerable investment involved in producing hybrid plants, especially if in vitro interventions are required. It is thus important to be able to screen potentially valuable hybrids from any non-hybrids that may be recovered. A range of techniques with varying levels of sophistication are available for identifying hybrids.

VERIFICATION OF HYBRIDS

Techniques used to confirm hybrids can include morphological, cytological, biochemical, or molecular markers. Sometimes a combination of techniques may be used, the choice of which is based on available technologies, fitness-for-purpose, and sensitivity.

The chromosome complement of a species can be described by its karyotype, which includes details of the number, type, shape, and banding patterns of chromosomes. In many cases it is sufficient to only base the analysis on numbers of chromosomes and, therefore, plant breeders often use chromosome counts to identify hybrid or polyploid plants. Species can also be identified by their nuclear DNA content and flow cytometry is a technique for measuring the amount of nuclear DNA in plant cells. For example, diploid *Limonium sinuatum* has 16 chromosomes, *L. perezii* 14 chromosomes and their hybrid 2n = 15 (Morgan et al., 1998). Nuclear DNA contents for *L. perezii*, *L. sinuatum*, and the hybrid were 8.69, 6.42, and 7.59 pg, respectively (Morgan et al.,1998). In another cross between two *Limonium* species the situation was more complicated as both of the parents, *L. peregrinum* and *L. purpuratum,* and their hybrids, all had 24 chromosomes (Morgan et al., 1995). The existence of hybrids could be confirmed on the basis of leaf morphology as they approached maturity, but early confirmation of hybrids was made possible by flow cytometry. *Limonium peregrinum* has a mean 2C nuclear DNA content of 13.98 pg, the hybrid 16.81 pg, and *L. purpuratum* 19.37 pg (Morgan et al., 1995). This is a clear example of closely related species having different nuclear DNA content despite their similar chromosome number. In both these *Limonium* crosses symmetrical hybrids with intermediate chromosome number (*L. perezii* × *L. sinuatum*) and nuclear DNA content were produced. The morphology of the hybrids was also intermediate to that of the parents, e.g., the *L. perezii* and *L. sinuatum* hybrid had the club-shaped leaves of *L. perezii* with the "wavy" leaf margins of *L. sinuatum*. The intergeneric hybrid *Sandersonia aurantiaca* × *Littonia modesta* (Morgan et al., 2001a) was also symmetrical with respect to chromosome numbers and nuclear DNA content, but differences in leaf shape were less obvious than in *Limonium* hybrids; the partial fusion of tepals in flowers of the hybrid contrasted with complete fusion of tepals in *Sandersonia* and complete separation in *Littonia* (Morgan et al., 2001a).

In contrast to *Limonium* or *Sandersonia*, *Gentiana* is a much more genetically diverse and cosmopolitan genus, with natural introgression occurring within close communities. Therefore, some interspecific gentian hybrids cannot be identified using either chromosome number or flow cytometry due to very similar nuclear DNA contents. Recently developed molecular markers may be used to distinguish hybrids from closely related gentian species (Pathirana et al., 2011) or, if needed, morphological markers become more useful as the plants mature. With crosses between more distant species, e.g., *Gentiana triflora* × *G. lutea*, both chromosome numbers and nuclear DNA contents can be used to distinguish hybrid plants from their parents (Morgan, 2004).

OVERCOMING CHALLENGES ASSOCIATED WITH HYBRIDS

Hybrids are generated to form new genetic combinations but will include genetic material conferring both desired and undesired traits. Typically the hybrids will be backcrossed to one parent species to eliminate undesirable traits. In many cases it is a straightforward matter to begin a programme of backcrossing to incorporate the hybrid plants into breeding programs. However, this process of backcrossing may be complicated with infertility or various manifestations of incompatibility limiting breeding opportunities.

In many wide crosses the hybrid plants are infertile. This infertility can arise from poor pairing (homology) between the two sets of parental chromosomes or an uneven number of chromosomes, both of which result in the hybrid failing to produce viable pollen and egg cells (gametes), though other factors may also contribute to infertility. The *Limonium* hybrids described previously provide examples, where *Limonium sinuatum* has 2n = 16 chromosomes, *L. perezii* 2n = 14, and their infertile hybrid 2n = 15 (Morgan et al., 1998). In *L. peregrinum* × *L. purpuratum* the hybrids have 24 chromosomes (Morgan et al., 1995). Meiosis appears normal and pollen appears to be viable, but it won't germinate. Therefore backcross hybrids were produced using the hybrid as a seed parent. The reason for failure of hybrid pollen germination in this example is not known.

Infertility in interspecific hybrids is typically overcome by chromosome doubling to generate (allo-)polyploid plants. Chromosome doubling can be achieved in a number of manners, but in essence plant tissues with dividing cells are treated with a compound such as colchicine or oryzalin that interferes with spindle formation at cell division. The spindle separates the two sets of chromosomes in cells that are about to divide. If the chromosomes are not separated the resultant cell will have twice the number of chromosomes (is polyploid), and regeneration of a new plant from one of the cells with double the chromosome numbers will result in an allopolyploid (polyploid plant based on two species), thus restoring fertility to the hybrid. In vitro chromosome doubling has been applied to a range of genera. In our lab we typically transfer the plant material to proliferation medium and, when proliferating well, it is transferred to the same medium supplemented with oryzalin. The plant material remains on this medium for up to 4 weeks, and is then transferred to fresh medium lacking the oryzalin to recover and regenerate new tissues. The oryzalin treatment is stressful on the plant material, and there is likely to be considerable death and necrosis of the treated tissues. Nevertheless, when oryzalin is removed, new shoots can be regenerated. After two or three subculture cycles the plant material can be screened for polyploidy in the newly regenerated tissues using flow cytometry. After deflasking it may be possible to quickly screen plants in the

greenhouse for polyploidy on the basis of leaf thickness, leaf colour or stomata size. In our lab this protocol has been used with slight modifications for chromosome doubling in a range of genera, e.g., *Gentiana* (Morgan et al., 2003; Pathirana et al., 2011), *Limonium* (Morgan et al., 2001b), and *Zantedeschia* (unpublished data). In the example of *L. perezii* × *L. sinuatum,* chromosome doubling resulted in a fertile tetraploid plant with 30 chromosomes (Morgan et al., 2001b). The tetraploid hybrid was backcrossed to *L. perezii* to give a triploid hybrid, with plants produced using embryo rescue. The triploid plants were partly fertile, with further hybrids produced using embryo rescue (Morgan et al., 2001b).

Incorporating the newly created tetraploid hybrids into breeding programmes can be difficult if the hybrids are isolated from their (diploid) parents because of their increased ploidy (it can be difficult to hybridise diploid plants with tetraploids, even within the same species). Backcross hybrids (triploid) can be produced using embryo rescue to bypass the ploidy barrier as described previously. Another solution to this problem is chromosome doubling of the parent to produce a tetraploid (autopolyploid), which is then crossed with the allopolyploid hybrid, with further breeding occuring at this elevated ploidy level. This approach has been successfully applied to producing hybrids in genera where species occur at a range of ploidy levels, e.g., *Solanum*. *Solanum* species occur at ploidy levels from diploid to hexaploid, and most potato cultivars are tetraploid. Chromosome doubling of diploid species improved compatibility with cultivated potatoes (Jansky, 2006).

Hybrids may exhibit a range of traits that make them inherently weak or unable to contribute to further generations. Albinism has been documented in a number of cases, e.g., *Zantedeschia* hybrids, in which plastid development was inhibited by plastome-genome incompatibility (Yao et al., 1994). In interspecific *Limonium* hybrids, Morgan et al. (1998) described pale foliage which may be the result of a similar incompatibility reaction, though this was not specifically investigated. Backcrossing the hybrids to either parent resulted in dark green foliage, with plants of normal appearance (Morgan et al., 1998). Hence backcrossing appears to present a possible solution to overcome this problem.

In other interspecific crosses, the hybrids may have inherent weaknesses resulting in early senescence and plant death. For instance, the hybrids between *Gentiana triflora* and *G. lutea* (Morgan, 2004), as well as *G. triflora*, and *G. asclepiadea* (unpublished data), although green and apparently normal, have proven very difficult to grow after transfer to the greenhouse. Hybrid plants that grow poorly and display symptoms suggestive of pathogen attack or other stress may be subject to "hybrid necrosis." Hybrid necrosis is a phenomenon that has received little attention in the scientific literature and is poorly understood. A greater understanding of the underlying mechanism(s) will undoubtedly enable access to an increased range of interspecific hybrids in the future.

CONCLUSIONS

Modern techniques and tools for interspecific hybridisation create exciting new opportunities for developing novel plant genotypes by overcoming the barriers that define species. Hybridisation between widely separated species increases the likelihood of outcomes such as infertility or hybrid weakness, but in many cases there are solutions to these challenges. In spite of these challenges, the value of distant hybridisation to plant breeding ensures it will remain a valued tool in plant breeding programs.

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LITERATURE CITED

Abbott, R.J, A.C. Brennan, J.K. James, D.G. Forbes, M.J. Hegarty, and **S.J. Hiscock.** 2009. Recent hybrid origin and invasion of the British Isles by a self-incompatible species, Oxford Ragwort (*Senecio squalidus* L., Asteraceae). Biol. Invasions 11:1145–1158.

Burge, G.K., E.R. Morgan, J.R. Eason, G.E. Clark, J.L. Catley, and **J.F. Seelye.** 2008. *Sandersonia aurantiaca*: Domestication of a new ornamental crop. Sci. Hortic.-Amsterdam 118:87–99.

Diederichsen, E., and **M.D. Sacristan.** 1988. Interspecific hybridisations in the genus *Brassica* followed by in-ovule embryo culture. Crucif. Newslett. 13:20–21.

Hajjar, R., and **T. Hodgkin.** 2007. The use of wild relatives in crop improvement: A survey of developments over the last 20 years. Euphyt. 156:1–13.

Heesacker, A.F., E. Bachlava, R.L. Brunick, J.M. Burke, L.H. Rieseberg, and **S.J. Knapp.** 2009. Karyotypic evolution of the common and silverleaf sunflower genomes. Plant Genome 2(3):233–246.

Hilu, K. 1993. Polyploidy and the evolution of domesticated plants. Amer. J. Bot. 80(12):1494–1499.

Jansky, S. 2006. Overcoming hybridization barriers in potato. Plant Breeding 125:1–12.

Mayr, E. 1942. Systematics and the origin of species. Columbia University Press, New York.

Morgan, E.R., G.M. Timmerman-Vaughan, A.J. Conner, W.B. Griffin, and **R.A. Pickering.** 2011. Plant interspecific hybridisation: outcomes and issues at the intersection of species. Plant Breed. Rev. 34:161–220.

Morgan, E.R. 2004. Use of *in ovulo* embryo culture to produce interspecific hybrids between *Gentiana triflora* and *Gentiana lutea*. N.Z J. Crop Hort. Sci 32:343–347.

Morgan, E.R., G.K. Burge, J.F. Seelye, J.E. Grant, and **M.E. Hopping.** 2001a. Wide crosses in the Colchicaceae: *Sandersonia aurantiaca* × *Littonia modesta*. Euphyt. 121:343–348.

Morgan, E.R., G.K. Burge, and **J.F. Seelye.** 2001b. *Limonium*. New options for a well known genus. Acta Hort. 552:39–42.

Morgan, E.R., G.K. Burge, J.F. Seelye, J.A. Grant, and **M.E. Hopping.** 1995. Interspecific hybridisation between *Limonium peeigrinum* and *Limonium purpuratum*. Euphyt. 83:215–224.

Morgan, E.R., G.K. Burge, J.F. Seelye, M.E. Hopping, and **J.E. Grant.** 1998. Production of interspecific hybrids between *Limonium perezii* and *Limonium sinuatum*. Euphyt. 102:109–115.

Pimentel, D., C. Wilson, C. McCullum, R. Huang, P. Dwen, J. Flack, Q. Tran, T. Saltmam, and **B. Cliff.** 1997. Economic and environmental benefits of biodiversity. BioSci. 47:747–757.

Sukhvibul, N., and **J.A. Considine.** 1993. Medium and long term storage of *Anigozanthos manglesii* (D. Don) pollen. N.Z. J. Crop Hort. Sci. 21:343–347.

U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Japan. J. Bot. 7:389–452.

van Harten, A.M. 1998. Mutation breeding: theory and practical applications. Cambridge University Press, Cambridge.

Van Tuyl, J.M. 1997. Interspecific hybridization of flower bulbs: A review. Acta Hort. 430:465–476.

Van Tuyl, J.M., and **M.J. De Jeu.** 1997. Methods for overcoming interspecific crossing barriers, pp. 273–292. In: Shivanna, K.R., and V.K. Sawhney (eds.), Pollen biotechnology for crop production and improvement. Cambridge University Press, London.

Van Tuyl, J.M., H.S. Chi, B.C.M. van Kronenburg, and **B. Meijer.** 1997. Interspecific lily hybrids: a promise for the future. Acta Hort. 430:539–544.

Van Tuyl, J.M., and **K.B. Lim.** 2003. Interspecific hybridisation and polyploidisation as tools in ornamental plant breeding. Acta Hort. 612:13–22.

Van Tuyl, J.M., M.P. Van Dien, M.G.M. Van Creij, T.C.M. Van Kleinwee, J. Franken, and **R.J. Bino.** 1991. Application of in vitro pollination, ovary culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci. 74:115–126.

Vervaeke, I., E. Parton, L. Maene, R. Deroose, and **M.P. De Proft.** 2002. Pollen tube growth and fertilization after different in pollination techniques of *Aechmea fasciata.* Euphyt. 124:75–83.

Yao, J-L., D. Cohen, and **R.E. Rowland.** 1994. Plastid DNA inheritance and plastome-genome incompatibility in interspecific hybrids of *Zantedeschia* (Araceae). Theor. Appl. Genet. 88:255–260.

Zou, X., D.W. Fountain, and **E.R. Morgan.** 2001. Anatomical and morphological studies of seed development in *Sandersonia aurantiaca* (Hook.). S. Afr. J. Bot. 67:183–192.